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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02005452.4

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Use of EGFR transactivation inhibitor in human cancer

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Use of EGFR transactivation inhibitors in human cancer

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Use of EGFR transactivation inhibitors in human cancer

Description

The present invention relates to the use of a compound which is capable of inhibiting activation of growth-factor receptors of the EGFR-family for the prevention or treatment of processes associated with increased G-protein mediated signal transduction.

Signaling through receptor tyrosine kinases (RTK) is involved in the regulation of fundamentally important cellular processes and its upregulation has been shown to be connected to hyperproliferative diseases such as cancer. G protein-coupled-receptors (GPCR) constitute the largest group of cell surface receptors controlling multiple signaling cascades and their biological outcome. Recently, crosstalk between members of both receptor families has been described that connects the multitude of different stimuli via GPCR ligands with the signaling capability of RTKs such as the epidermal growth factor receptor (EGFR), see e.g. WO01/12182. The signaling mechanism which involves shedding of growth-factor precursors by a metalloprotease led us to propose the model of the triple-membrane-passing signal (TMPS) pathway.

Our object was to investigate the physiological processes that are regulated by the TMPS pathway and its significance for pathophysiological phenomena such as neoplastic progression. Therefore, we screened human cancer cell lines for EGFR transactivation by stimulation with GPCR ligands, as well as inhibition of metalloproteases. Specifically, we investigated growth factor-stimulated events in signal transduction and in defined physiological processes.

Our results show that EGFR phosphorylation as well as downstream signaling events such as the recruitment of adapter proteins and phosphorylation of the mitogen-activated protein kinase occur after stimulation with GPCR ligands and are downregulated by the metalloprotease inhibitor batimastat. Additionally, basal phosphorylation of the EGFR is batimastat sensitive. Furthermore, we revealed that EGFR transactivation is part of the regulatory system which modulates cell cycle progression and cell proliferation. The TMPS pathway is also able to promote anti-apoptosis, cell migration and invasivity.

While somatic cells require external mitogenic signals, cancer cells are characterized by abnormal growth behaviour due to autocrine production of mitogenic factors. As many of these are GPCR ligands, EGFR transactivation constitutes a mechanism for cancer progression by deregulation of cell proliferation and suppression of cell death. Inhibition of this pathway is therefore a promising strategy for the treatment of cancer.

Thus, a subject matter of the present invention is the use of a compound which is capable of inhibiting activation of a growth-factor receptor of the EGFR family for the manufacture of an agent for the prevention or treatment of processes selected from cell proliferation, cell migration, invasivity and anti-apoptosis in a disorder, which is associated with increased G-protein mediated signal transduction.

Surprisingly, it was found that inhibition of growth-factor receptor activation caused by increased G-protein mediated signal transduction leads to an inhibition of cancer progression, particularly cell migration and invasivity, as well as to an inhibition of anti-apoptosis. Thus, inhibitors of the GPCR-induced growth-factor receptor activation are suitable for the manufacture of medicaments for the prevention or treatment of specific indication of hyper-proliferative diseases associated with cell-proliferation,

cell migration, invasivity and/or anti-apoptosis and to re-establish control of these phenomena in the treated cells or organisms, respectively.

The growth-factor receptor is preferably EGFR or another member of the EGFR family, such as HER-2, HER-3 or HER-4, but also other growth-factor receptors, particularly receptor tyrosine kinases may be inhibited.

The compound may act on a growth-factor receptor ligand precursor, which is preferably a membrane-associated molecule. In a particularly preferred embodiment the growth-factor ligand precursor is pro-HB-EGF which is cleaved to HB-EGF by a protease. Other preferred examples of growth-factor receptor ligands which are cleaved from precursors are other members of the EGF family, such as TGF , amphiregulin, epi-regulin, EGF, ß-cellulin and members of the heregulin/NDF family including isoforms thereof.

An example of a compound, which acts on a growth-factor receptor ligand precursor, is CRM197, a catalytically inactive form of the diphteria toxin, which specifically binds to pro-HB-EGF and which is capable of blocking the processing of pro-HB-EGF. A further example is an antibody, which is capable of binding to pro HB-EGF and which thereby blocks its processing.

In a further embodiment the compound acts on a metalloprotease, particularly a membrane-associated metalloprotease, e.g. a protease of the ADAM family. Inhibition of the protease leads to a blocking of the processing of growth-factor receptor ligand precursors and thus results in an inhibition of growth-factor receptor activation. Preferred examples of inhibitors of protease activity are batimastat (BB-94), marimastat, TAPI and TIMP-1-2-3 or -4, particularly TIMP-3.

In a still further embodiment the compound may act on the growth-factor receptor itself. For example, the compound may bind to the growth-factor

receptor and thereby inhibit transactivation. An example of such a compound is the EGFR-inhibitor AG1478. Further, growth-factor receptor-binding antibodies may be used.

It should be noted that the present invention relates to a targeted inhibition of cellular signal pathways "downstream" of the EGFR transactivation in cancer cells, particularly in human cancer cells.

The disorder to be treated or prevented is associated with and preferably caused by increased G protein-mediated signal transduction. This increased G protein-mediated signal transduction may be associated with or caused by a pathological increase in the activity of a G protein and/or a G protein-coupled receptor (GPCR). It should be noted that the disorders which are prevented or treated according to the present invention can be delimited from other hyperproliferative disorders having an enhanced growth-factor receptor expression in that a transactivation of growth-factor receptor expression via G protein signal pathways takes place. More particularly, the disorder is a cancer, such as a colon, kidney, liver, bladder, pancreatic, prostate, gastric, breast, lung, thyroid, pituitary, adrenal or ovarian tumor or glioblastoma.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g. for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture

assays. For example, a dose can be form[illegible]ated in animal models to achieve a circulating concentration range that i[illegible]udes the IC50 as determined in cell culture (i.e. the concentration of th[illegible]est compound which achieves a half-maximal inhibition of the growt[illegible]actor receptor activity). Such information can be used to more acc[illegible]tely determine useful doses in humans. The dose ratio between tox[illegible]and therapeutic effects is the therapeutic index and it can be expres[illegible]s the ratio between LD50 and ED50. Compounds which exhibit high [illegible]apeutic indices are preferred. The exact formulation, route of admini[illegible]tion and dosage can be chosen by the individual physician in view of th[illegible]atient's condition (see e.g. Fingl et al., 1975, in "The Pharmacological B[illegible]f Therapeutics", Ch. 1, p. 1). Dosage amount and interval may be adj[illegible] individually to provide plasma levels of the active moiety which are [illegible]cient to maintain the receptor modulating effects, or minimal effective [illegible]centration (MEC). The MEC will vary for each compound but can be es[illegible]ed from in vitro data, e.g. the concentration necessary to achieve a [illegible]% inhibition of the receptor using the assays described herein. C[illegible]unds should be administered using a regimen which maintains plasm[illegible]e above the MEC for 10-90% of the time, preferably between 30-90[illegible] most preferably between 50-90%. Dosages necessary to achieve [illegible]C will depend on individual characteristics and route of administrat[illegible] cases of local administration or selective uptake, the effective local [illegible]tration of the drug may not be related to plasma concentration.

The actual amount of composition [illegible]istered will, of course, be dependent on the subject being trea[illegible] the subject's weight, the severity of the affliction, the manner o[illegible]istration and the judgement of the prescribing physician. For batim[illegible], and other compounds e.g. a daily dosage of 1 to 200 mg/kg, partic[illegible] 10 to 100 mg/kg per day is suitable.

Further, the invent[illegible]on [illegible]elates to a [illegible]ethod for identifying and/or characterizing an inhi[illegible]itor [illegible] processes [illegible]cted from cell proliferation, cell migration, invasivity and anti-apoptos[illegible] in a disorder associated with increased G-protein med[illegible] signal tra[illegible]uction, comprising: determining the effect of a test comp[illegible]und on the [illegible]sactivation of a growth-factor receptor of the EGFR famil[illegible]. [illegible]his meth[illegible] [illegible]roferably a screening method which comprises a f[illegible] assay f[illegible] [illegible] proliferation, cell migration, invasivity and/or anti-ap[illegible] s, e.g. as [illegible]ibed in the examples. The test compounds may be se[illegible] from lo[illegible] [illegible]olecular weight compounds, peptides and protein[illegible], pa[illegible]ticularly anti[illegible]s or antibody fragments.

Further, the inventio[illegible] s[illegible] be explain[illegible] more detail by the following examples:

EXAMPLE

1. METHODS

1.1 Cell lysis, immu[illegible]ation and [illegible]noblotting

Prior to lysis, cells gro[illegible] to [illegible]0% conf[illegible] [illegible] were treated with inhibitors and agonists and ly[illegible]ed f[illegible] [illegible]0 min o[illegible] in buffer containing 50 mM HEPES pH 7.5, 150 m[illegible] NaCl, 1% [illegible] X-100, 1 mM EDTA, 10% glycerol, 10 mM sod[illegible]um [illegible]phosphat[illegible] [illegible]M sodium orthovanadate, 10 mM sodium fluoride, 1 [illegible]M [illegible]henylme[illegible]lfonyl fluoride and 10μg/ml aprotinin. Lysates we[illegible]re [illegible]ared by c[illegible]fugation at 13000 rpm for 10 min at 4°C. Supernatant[illegible] [illegible]re dilute[illegible] [illegible] an equal volume of HNTG buffer and subsequen[illegible] immunopr[illegible]ated using the respective antibodies and 30μl [illegible] A-Sep[illegible] for 4 h at 4°C. Precipitates were washed three t[illegible] [illegible] 0.5 ml [illegible]G buffer, suspended in SDS sample buffer and s[illegible]je[illegible] [illegible]n gel ele[illegible]resis on 7.5% or 10% gels. Following SDS pa[illegible], [illegible] wer[illegible] [illegible]sferred to a nitrocellulose membrane and imm[illegible].

1.2 Invasion assay

Cell invasion assays w[illegible] performed [illegible]oyden chambers. Serumfree medium containing th[illegible]attractan[illegible] tested is added to the lower well of a Boyden cha[illegible]atrigel c[illegible] filter is placed over the lower well of the Boyden cha[illegible]nd is se[illegible] with a gasket. The cells are preincubated with the [illegible] for 20 [illegible]and then added to the upper wells of the chambers [illegible]ree me[illegible]. The chambers are incubated for 6 h to overnight [illegible]fied 7% [illegible] 37°C incubator. Finally, cells are wiped from the [illegible]ace with [illegible]ton tip swab and the cells on the lower side are stai[illegible] counted [illegible]r the microscope.

1.3 Migration assay

Cell migration assays [illegible] perform[illegible] Boyden chambers containing polycarbonate mem[illegible] pore s[illegible] described above.

1.4 Cell wounding ass[illegible]

Cells were grown to [illegible] in six [illegible]tes in standard medium and analysed using [illegible]cratch [illegible] method. Cells were gently scraped with a plastic [illegible] medi[illegible]s removed, and cells were washed twice with PB[illegible]free Me[illegible]as added and the cells were permitted to migrate in[illegible]area of c[illegible] for 24 h to 48 h. Analysis was performed unde[illegible]scope.

1.5 Thymidine incorp[illegible]

Cells were seeded in [illegible]ates gr[illegible] 70% confluence, and then incubated in seru[illegible] for 24 [illegible]duce quiescence. Mitogenic stimuli were then [illegible]e 0, [illegible]equired pre-treatment with inhibitors. After 18 h [illegible]yl-^{3}H)[illegible]ne was added to each well.

Four hours later the [illegible] media was [illegible]ved, cells were washed with PBS, fixed with ice [illegible] trichlor[illegible] acid. Labelled material was then solubilized and [illegible] associated ra[illegible]ivity was then quantified by liquid scintillation co[illegible].

1.6 Detection of Apo[illegible]s

Apoptosis was meas[illegible] by f[illegible]ow cyto[illegible]. Cells were seeded in six well plates and starved. A[illegible] was indu[illegible] by anti-CD95 antibody, at the same time inhibitors [illegible] afterwards m[illegible]ns were added after 24 h. At the end of the cultu[illegible] cells we[illegible]shed in PBS, resuspended in hypotonic buffer cont[illegible]g 50μg/ml p[illegible]m iodide, kept for 2 h at 4°C in the dark, and anal[illegible] by [illegible]ow cyto[illegible]. The percentage of apoptotic cells was determined [illegible] va[illegible]ating hy[illegible]ploid nuclei after proper gating on DNA content.

2. RESULTS

Investigation of the [illegible]ol[illegible]ical pro[illegible] that are regulated by EGFR transactivation via th[illegible] [illegible]le-membrane [illegible]ing signal (TMPS)-pathway:

1. Several human [illegible]er cell lines s[illegible] [illegible]GFR transactivation by GPCR ligands as we[illegible] inhibi[illegible]tion of [illegible]proteases.

2. Our results sh[illegible] that [illegible]GFR phos[illegible]lation as well as downstream signaling even[illegible] [illegible]ch as the re[illegible]ent of adapter proteins and phosphorylati[illegible] of the mitogen [illegible]d protein kinase occur after stimulation [illegible] GPC[illegible] ligand[illegible] are downregulated by the metalloprote[illegible] in[illegible]itor [illegible]stat. Additionally, basal phosphorylat[illegible] th[illegible] EGFR is [illegible]tat sensitive.

3. EGFR transa[illegible]on via the [illegible]PS pathway promotes cell proliferation, [illegible]optosis, cell [illegible]ation and invasion.

The results are sum[illegible]d [illegible] Fig. 1-7 [illegible]n Table 1.

3. CONCLUSIONS

Cancer cells are c[illegible]terized by ab[illegible]al growth behaviour due to autocrine productio[illegible] [illegible]ito[illegible]enic fac[illegible] As many of these are GPCR ligands, EGFR trans[illegible]tion constitu[illegible] likely mechanism for cancer progression by dere[illegible]tion of cell pr[illegible]ation and suppression of cell death. Inhibition of t[illegible] pathway is the[illegible] a promising strategy for the treatment of cancer.

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Claim

1. Use of a compound which is capable of inhibiting activation of a growth-factor receptor of the EGFR family for the manufacture of an agent for the prevention or treatment of processes selected from cell proliferation, cell migration, invasivity and anti-apoptosis in a disorder, which is associated with increased G-protein mediated signal transduction.

2. The use of claim 1 wherein the growth-factor receptor is EGFR.

3. The use of claim 1 or 2 wherein the compound acts on a growth-factor receptor ligand precursor.

4. The use of claim 3 wherein the growth-factor receptor ligand precursor is EGF or an EGF-like ligand.

5. The use of claim 1 or 2 wherein the compound acts on a metalloprotease.

6. The use of claim 5 wherein the compound directly inhibits the protease activity.

7. The use of claim 1 or 2 wherein the compound acts on the growth-factor receptor.

8. The use of any one of claims 1 to 7 wherein the agent is a pharmaceutical composition comprising at least one pharmaceutically acceptable carrier, diluent and/or adjuvant.

9. The use of any one of claims 1 to 8 wherein the disorder is cancer.

10. The use of any one of claims 1 to 9 wherein the cancer is a human cancer.

11. A method for identifying and/or characterizing an inhibitor of processes selected from cell proliferation, cell migration, invasivity and anti-apoptosis in a disorder associated with increased G-protein mediated signal transduction, comprising:
determining the effect of a test compound on the transactivation of a growth-factor receptor of the EGFR familiy.

12. The method of claim 11, wherein the test compound is selected from low-molecular weight compounds, peptides and proteins, particularly antibodies or antibody fragments.

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Abstract

The present invention relates to the use of a compound which is capable of inhibiting activation of a growth factor receptor of the EGFR-family for the prevention or treatment of processes associated with increased G-protein mediated signal transduction.

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Tyrosine phosphorylation of the EGFR and Shc occur after stimulation with GPCR ligands and are downregulated by BB94 and AG1478

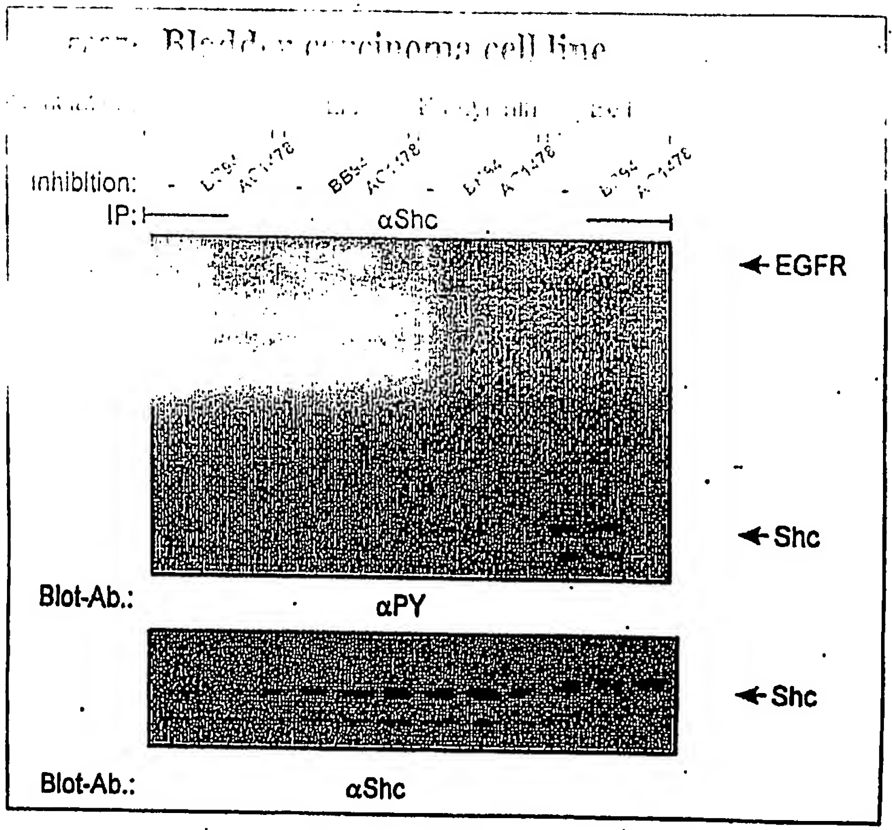


FIGURE 2

Phosphorylation of the mitogen-activated protein kinase after stimulation with GPCR ligands is downregulated by BB94 and AG1478

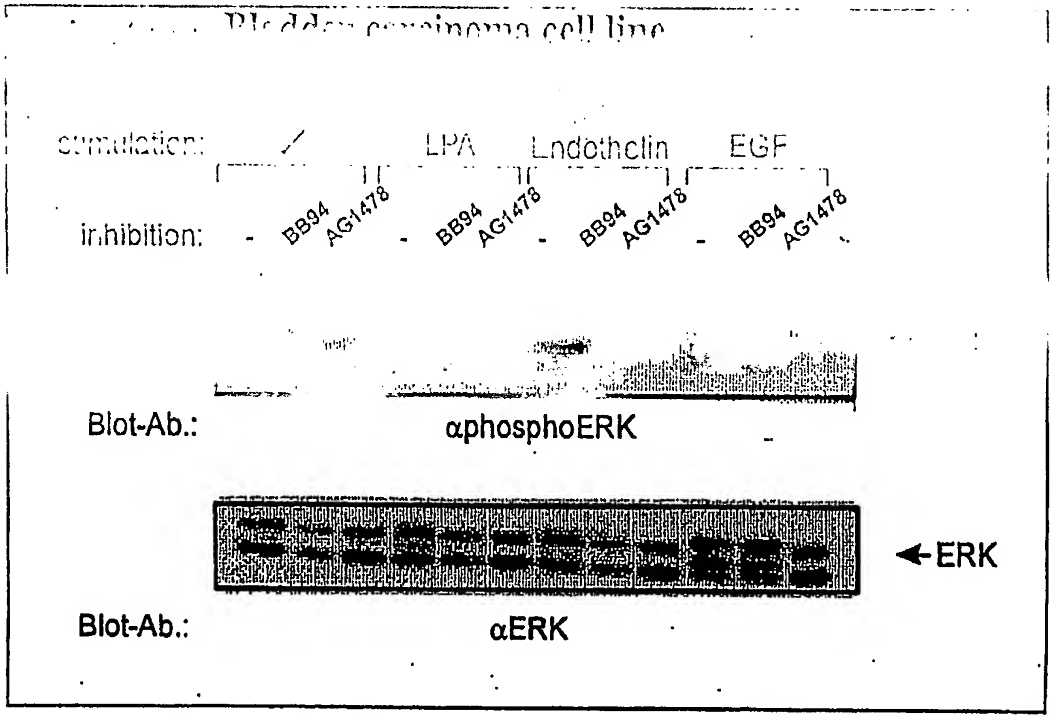
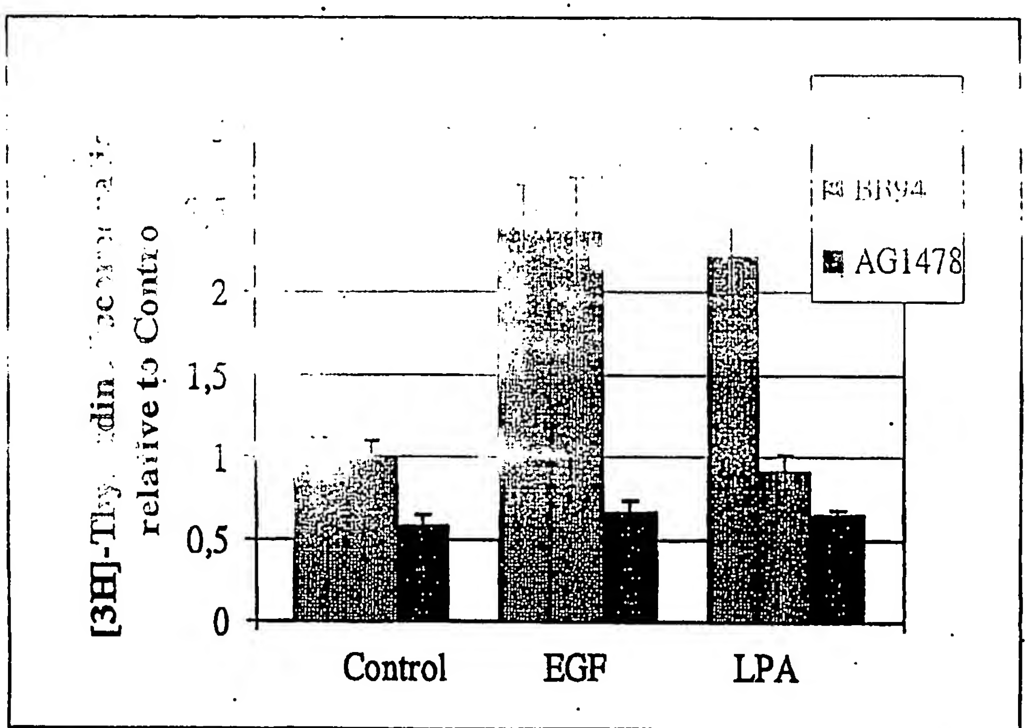


FIGURE 3

LPA induced proliferation in the lung cancer cell line H292 can be inhibited by BB94 and AG1478



Antiapoptotic effects induced by LPA in the bladder carcinoma cell lineTccSup can be inhibited by BB94 and AG1478

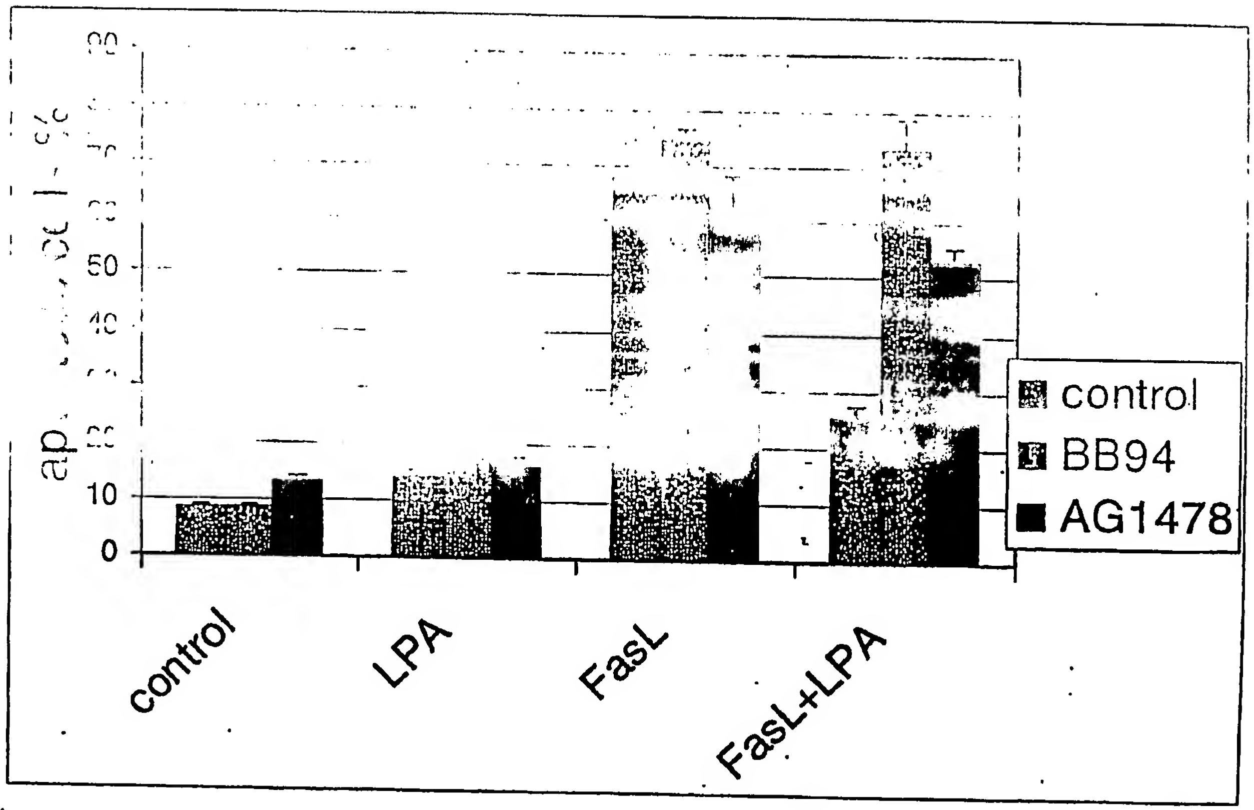


FIGURE 5

LPA induced migration in the kidney carcinoma cell line A498 can be inhibited by BB94 and AG1478

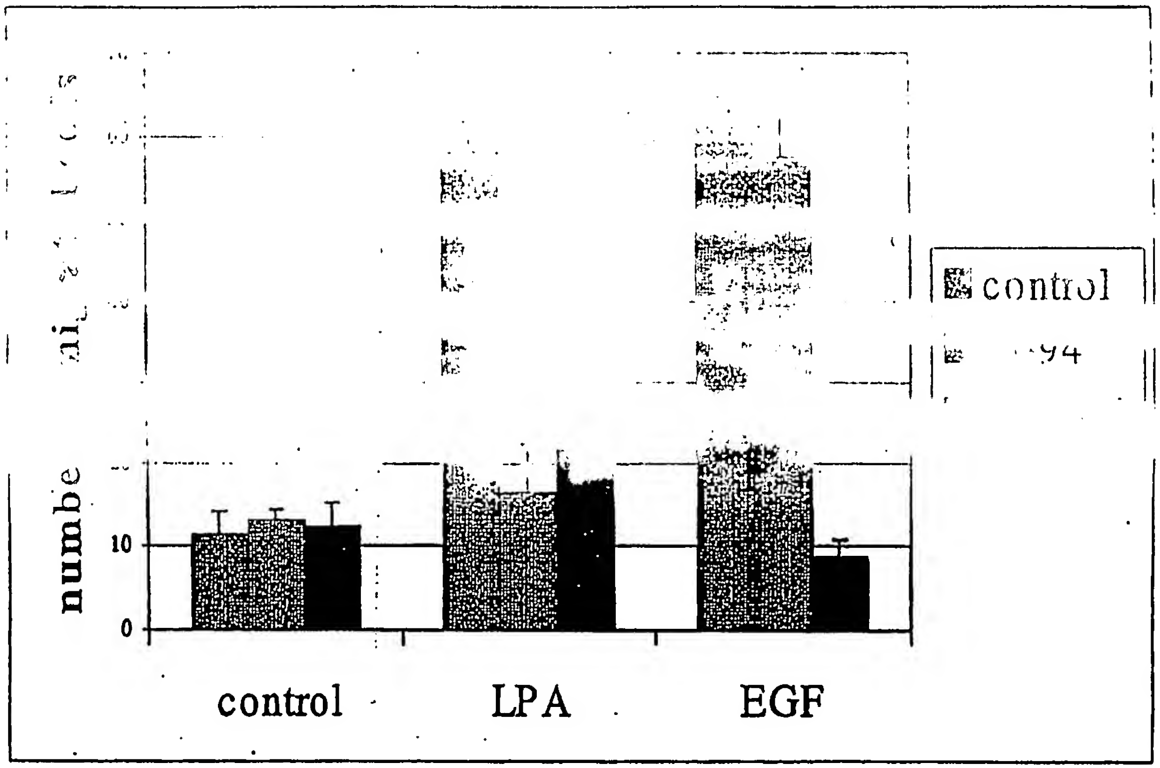


FIGURE 6

LPA induced wound closure in the kidney carcinoma cell line ACHN can be inhibited by BB94 and AG1478

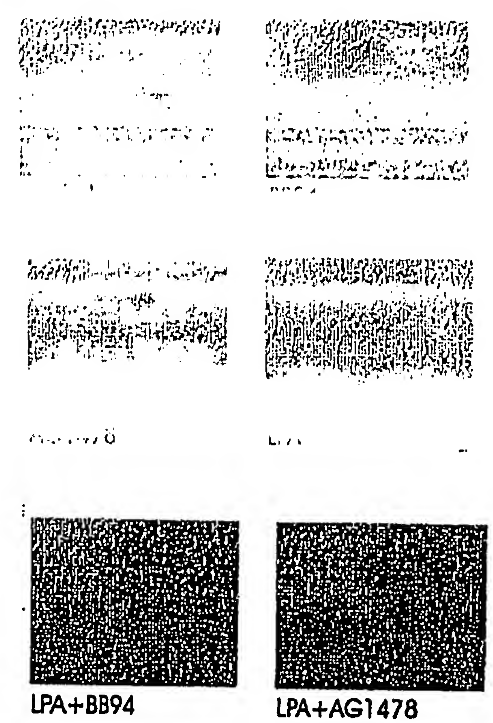


FIGURE 7

LPA promotes invasion in the kidney carcinoma cell line CaKi2 which can be diminished by BB94 and AG1478

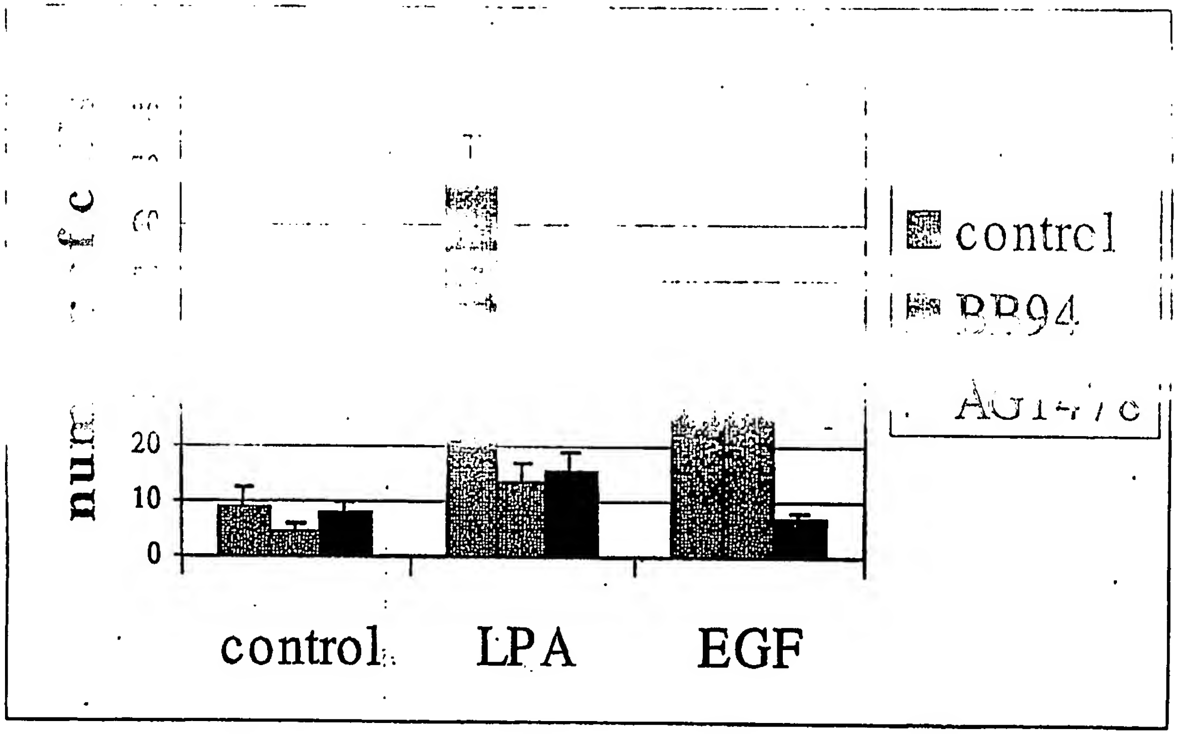


TABLE 1

Screening of human cancer cell lines for the physiological relevance of EGFR transactivation

[illegible]			[illegible] physiological relevance			
			[illegible]tion	[illegible]gration	invasion	antiapoptosis
[illegible]	human	kidney	n/d	n/d	n/d	n/d
A498	human	kidney	no	yes	yes	no
A704	human	kidney	no	n/d	n/d	n/d
SCABER	human	bladder	no	no	n/d	no
HT1376	human	bladder	no	no	n/d	no
TccSup	human	bladder	no	yes	yes	yes
H292	human	lung	yes	no	no	no

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